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Automated Ribotyping of Vancomycin-Resistant *Enterococcus faecium* Isolates

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Vancomycin-resistant *Enterococcus faecium* (VREF) strains represent an important threat in hospital infections in the United States and are found at high frequencies in both the community and farm animals in Europe. We evaluated automated ribotyping for interlaboratory reproducibility by using the restriction enzymes *Eco*RI and *Bam*HI and compared ribotyping to both amplification of fragment length polymorphism (AFLP) analysis and multilocus sequence typing (MLST) to assess its discriminatory power and capacity for the identification of epidemiologically important strains. Of 19 (*Eco*RI) and 16 (*Bam*HI) isolates tested in duplicate in two laboratories, 18 (95%) and 16 (100%), respectively, showed reproducible ribotypes. These high reproducibility rates were obtained only after manual refinement of the automated fingerprint analysis. A group of 49 VREF strains initially selected to represent 32 distinct AFLP types were separated into 28 *Eco*RI ribotypes, 25 *Bam*HI ribotypes, and 28 sequence types. Ribotyping with *Eco*RI and *Bam*HI was able to discern the host-specific genogroups recently disclosed by AFLP typing and MLST and to distinguish most strains containing the *esp* gene, a marker specific for strains causing hospital outbreaks. An expandable ribotype identification library was created. We recommend *Eco*RI as the enzyme of choice for automated ribotyping of VREF strains. Given the high level of discrimination of VREF strains, the high rate of interlaboratory reproducibility, and the potential for the identification of epidemiologically important genotypes, automated ribotyping appears to be a very valuable approach for characterizing VREF strains.

Vancomycin-resistant *Enterococcus faecium* (VREF) strains represent an important threat in hospital infections in the United States and are found at high frequencies in both the community and farm animals in Europe (13, 29). VREF strains are often multidrug resistant, a fact which makes infections with VREF strains difficult to treat. As VREF strains often cause outbreaks in hospitals, genetic characterization techniques are needed to track their dissemination.

Numerous genetic typing methods are currently used to type *E. faecium* isolates, including the "gold standard" pulsed-field gel electrophoresis (PFGE) (17) and the recently developed amplified fragment length polymorphism (AFLP) analysis (1, 28) and multilocus sequence typing (MLST) (12a). Interestingly, by AFLP typing, four major genogroups were distinguished and VREF strains present in the different genogroups exhibited host specificity (28). Importantly, VREF strains causing colonization and infections in hospitalized patients (genogroup C) are distinct from strains causing colonization in nonhospitalized persons (genogroup A). The ability to efficiently distinguish these two genogroups has important practical consequences with respect to screening for VREF strains upon hospitalization and implementation of infection control measures. Moreover, VREF strains causing hospital outbreaks appear to represent a distinct genetic subset of genogroup C that can be distinguished by AFLP analysis and that is characterized by the presence of the *esp* gene (27). This finding makes it possible to rapidly identify, by use of genotypic fingerprinting methods, VREF strains with potentially increased epidemic capacities.

Major limitations of most typing methods, including the AFLP method, include a lack of standardization, the need for skilled personnel, and significant hands-on time to generate the data. Automated ribotyping, performed by using instruments called RiboPrinters (DuPont-Qualicon, Wilmington, Del.), automates most steps (from cell lysis to image analysis) in the ribotyping procedure. This method is currently the only automated fingerprinting method (5). Ribotyping, or rRNA gene restriction fragment analysis (10, 23), has been used on a manual basis with the genus *Enterococcus* both for identification to the species level (16, 21) and for strain characterization, mainly for the species *E. faecium* (2, 6,11, 14, 15, 18–20, 22).

To our knowledge, the interlaboratory reproducibility of automated ribotyping has never been reported in the scientific literature. Although ribotyping has been shown to be slightly less discriminatory than PFGE for typing *E. faecium* (12), the ease, speed, and standardization of automated ribotyping represent attractive advantages. The method could be very useful as a rapid screening method for investigating genetic relationships among isolates, for identifying strains of particular epidemiological relevance, and for constructing electronic databases of important ribotypes to allow remote identification through the Internet and tracking of these clones longitudinally and on a worldwide scale.

A characteristic of ribotyping which holds true for both manual and automated procedures is flexibility in terms of the restriction enzyme that can be used to generate the genetic fingerprints. This property represents an advantage, since dis-

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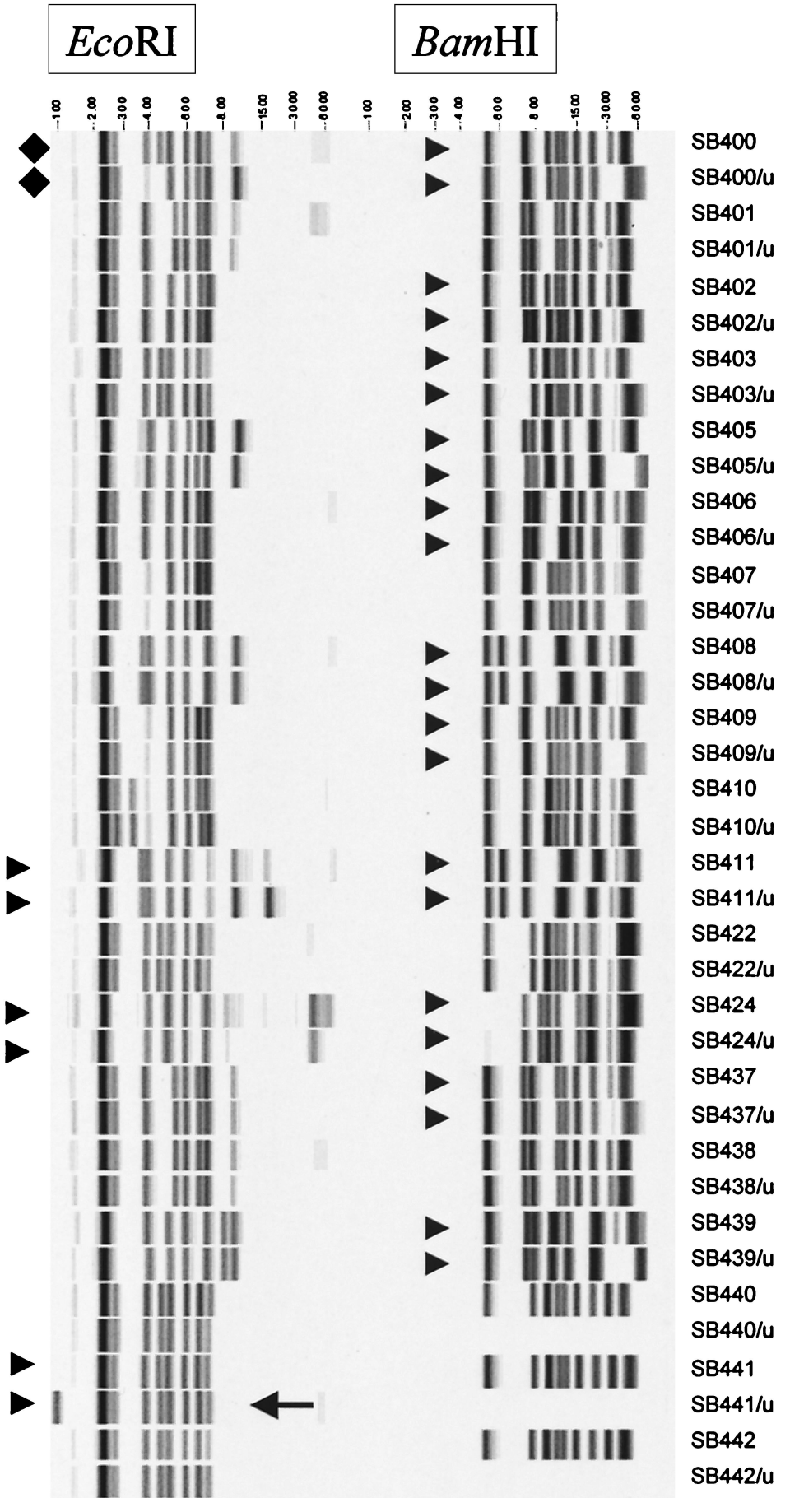
EcoRI
BamHI
SB400
SB400/u
SB401
SB401/u
SB402
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SB403
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SB405
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tinct enzymes can be suitable for distinct objectives, for example, discriminatory strain characterization versus species identification (4). However, the enzyme flexibility of ribotyping makes it necessary to carefully evaluate which enzyme is best suited for a given purpose. For interlaboratory comparisons, it is necessary that different laboratories use the same enzyme. A number of distinct enzymes have been used for ribotyping *E. faecium*, including *Eco*RI (6, 14), *Bam*HI (6, 18), *Hin*dIII and *Pvu*II (2), *Bsc*I (11), *Pst*I and *Ase*I (6), and *Xba*I (22), and there is no consensus on the best enzyme choice. Since *Eco*RI and *Bam*HI have been among the most widely used and are routinely used in our institutes, we decided to compare these two enzymes for their reproducibility, discriminatory power, and capacity for the identification of epidemiologically important strains. Furthermore, ribotype data were compared with both AFLP typing and MLST data to assess whether ribotyping is able to discern the same main genetic lineages among VREF strains as those previously disclosed by AFLP typing and MLST.

MATERIALS AND METHODS

Bacterial isolates. Four sets of isolates were included in this study. First, 49 isolates were derived from diverse origins, including human volunteers from the community ($n = 6$), hospitalized patients ($n = 17$), a pig ($n = 1$), cats ($n = 2$), dogs ($n = 3$), calves ($n = 8$), chickens (11), and a turkey ($n = 1$). The 49 isolates were chosen to fulfill three distinct criteria: (i) to be representative of diverse AFLP types within each of the four host-specific AFLP genogroups recently identified in *E. faecium* (28); (ii) to include strains belonging to the *esp*-positive lineage, found to be responsible for most epidemics in hospitals (27); and (iii) to include four groups of epidemiologically related isolates, three of which (two composed of three isolates each and one composed of two isolates) represented three hospital outbreaks and one of which (two isolates) came from a single patient. Most isolates were derived from the study of Willems et al. (28), and all 49 isolates were characterized by AFLP typing (28) and by MLST (12a).

Second, 40 *E. faecium* isolates, including VREF isolates and vancomycin-susceptible isolates, were used to determine the intra-RiboPrinter reproducibility of automated ribotyping by using enzyme *Bam*HI. These 40 isolates were derived from patients in distinct European hospitals as previously described (3). Third, 38 VREF isolates derived from hospital clinical isolates ($n = 18$) (3) and from stools of healthy individuals ($n = 20$) were analyzed with *Bam*HI and the University Medical Centre Utrecht (UMCU) RiboPrinter prior to this study. Finally, 45 *E. faecium* isolates of diverse origins were previously characterized with *Eco*RI and the Statens Serum Institute (SSI) RiboPrinter. The patterns of the last two sets of isolates were compared to those of the first isolate set for identification purposes.

Automated ribotyping. Strains were cultured on Columbia agar with 5% sheep red blood cells just before ribotyping. Automated ribotyping was performed by using the RiboPrinter microbial characterization system in accordance with the manufacturer's recommendations (5).

Ribotype categorization. Two approaches were compared in order to categorize the patterns into ribogroups, i.e., groups of strains showing ribotypes (fingerprints) considered to be identical. First, we considered the automatic categorization into ribogroups performed by the RiboPrinter software itself. This proprietary software (5) normalizes the digitized patterns; corrects for possible overall shifts in migration among the patterns by using an optimization parameter of 2%; and finally considers a threshold value of 93% overall similarity, as determined by using an algorithm similar to the Pearson correlation coefficient, to pool patterns into a single ribogroup. Second, manual categorization was implemented by visual inspection and manual correction of the RiboPrinter categorization. Correction was performed blindly (i.e., without information about the strain codes) by taking into account observed overall shifts in the patterns and the poor reproducibility for DNA fragments with molecular sizes of higher than 30 kb. A single obvious band difference was considered enough to distinguish ribogroups.

Cluster analysis. Normalized patterns were imported into the software BioNumerics 2.5 (Applied Maths, Sint-Martens-Latem, Belgium) by using an import script provided by DuPont-Qualicon. Band analysis was performed after an automatic band search by using the following parameters: minimum profiling parameter of 5.0% and gray-zone parameter of 5.0% relative to the maximal value, a minimum area parameter of zero, and a shoulder sensitivity parameter of zero. Clustering was performed by using the unweighted pair-group method with arithmetic averages (UPGMA) based on either the Pearson correlation (global pattern comparison) or the Dice similarity index (band-based analysis). Clustering was performed by using a 1% optimization parameter and a 1% band position tolerance.

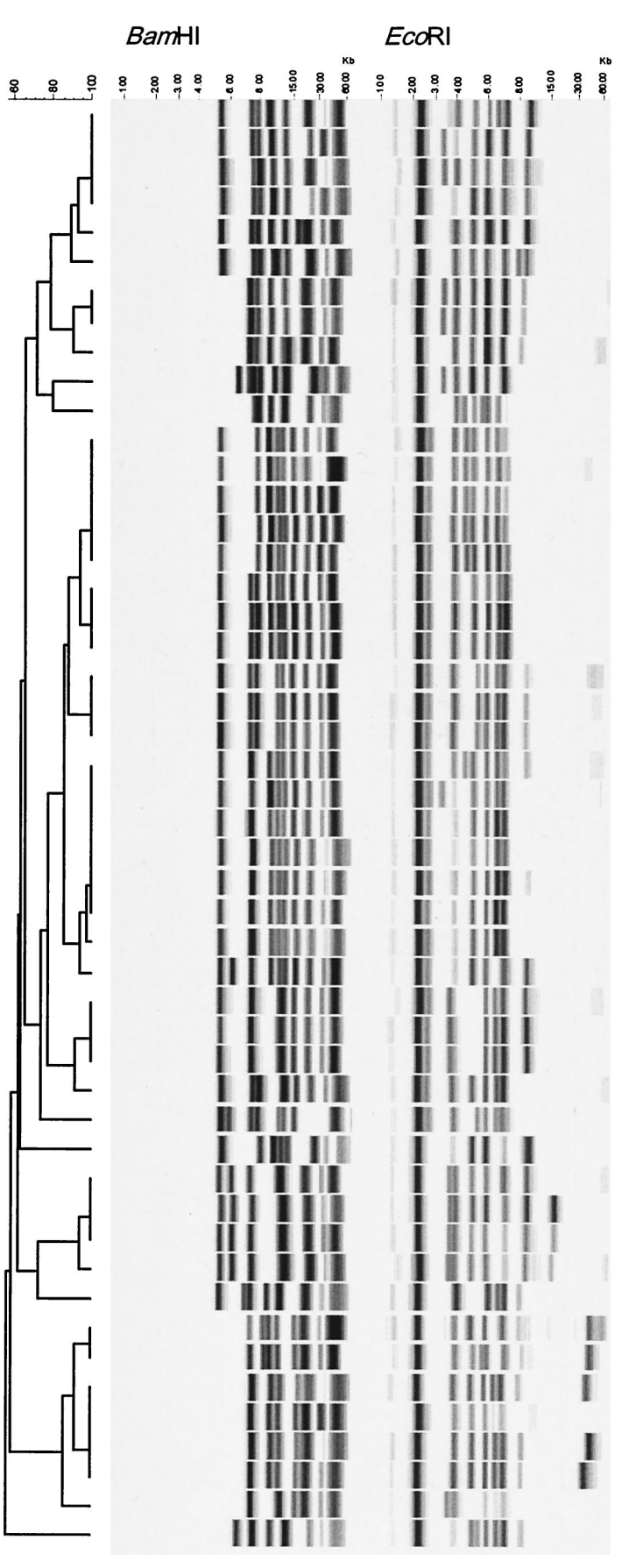
RESULTS

Technical results. The 49 isolates studied were ribotyped by using both restriction enzyme *Bam*HI and the RiboPrinter located at UMCU and restriction enzyme *Eco*RI and the RiboPrinter located at SSI. All strains were typeable with both enzymes. Ribotyping with enzyme *Eco*RI generated five to nine bands across the 49 isolates, ranging in size from 1.4 kb to 50 kb, with most bands distributed between 2.2 and 9.0 kb. With enzyme *Bam*HI, 6 to 10 bands were generated across the 49 isolates, ranging in size from 5.5 kb to 50 kb. Thus, the typical size ranges generated by the two enzymes were very distinct (Fig. 1 and 2). In particular, all *Bam*HI patterns showed bands in the 30- to 50-kb range, whereas only a few such bands were found after analysis with *Eco*RI. Overall, *Eco*RI patterns were easier to categorize than *Bam*HI patterns, which showed several wide and low-intensity bands (Fig. 1 and 2).

Reproducibility analysis. One of the objectives of this study was to assess the interlaboratory reproducibility of automated ribotyping. To this end, 19 strains were typed with *Eco*RI at UMCU and 16 strains were ribotyped with *Bam*HI at SSI. Data were sent electronically to UMCU for comparison (Fig. 1).

Among the 19 pairs of duplicate strains analyzed with *Eco*RI at the two institutes, 15 were automatically categorized into the same ribogroup by the RiboPrinter. Conversely, 18 of 19 duplicates fell into the same ribogroup after manual categorization. The three pairs that were reproducible upon manual categorization but not upon automated categorization corresponded to strains SB411, SB424, and SB441 (Fig. 1). The lack of reproducibility for strain SB441 upon automated categorization was due to a problematic run on the RiboPrinter, since the intrusion into the sample pattern of the DNA molecular weight marker band of 1 kb from the adjacent lane (Fig. 1) caused the misinterpretation of an extra band, as deduced from

FIG. 1. Interlaboratory comparison of automated ribotyping patterns obtained by using *Eco*RI and *Bam*HI restriction enzymes. The molecular size scale above the patterns is in kilobases. The letter "u" after a strain name corresponds to the second test of the strain (performed in Utrecht with *Eco*RI and in Copenhagen with *Bam*HI). The reproducibility of ribotyping with *Bam*HI was not tested for strains SB440, SB441, and SB442.The pairs of patterns that were not reproducible after automated categorization but that were reproducible after manual categorization are indicated with arrowheads. The only pair of patterns that was also not reproducible after manual categorization is indicated with diamonds. The arrow indicates the pattern into which a molecular size marker band of 1 kb was incorporated by the automated analysis of the RiboPrinter due to suboptimal electrophoretic migration.



	EcoRI	BamHI	Source	AFLP type	Genogroup	MLST type	esp
SB405	E-12	B-11	P	29	B	9	no
SB423	E-18	B-12	P	27	B	26	no
SB450	E-14	B-13	PF	5	B	15	no
SB458	E-16	B-12	P	10	B	8	no
SB461	E-26	B-14	P	10	B	8	no
SB439	E-15	B-25	PF	25	B	9	no
SB452	E-19	B-20	P	7	B	13	no
SB453	E-19	B-20	P	7	B	13	no
SB460	E-20	B-24	P	26	B	13	no
SB455	E-21	B-19	P	9	B	14	no
SB457	E-28	B-21	P	6	B	11	no
SB403	E-2	B-5	HP	22	C	17	yes
SB422	E-2	B-5	HP	24	C	16	yes
SB442	E-2	B-5	HP	20	C	20	yes
SB441	E-2	B-5	HP	20	C	20	yes
SB440	E-2	B-5	HP	20	C	20	yes
SB402	E-5	B-6	HP	19	C	17	yes
SB448	E-5	B-6	HP	19	C	17	no
SB449	E-5	B-6	HP	19	C	17	no
SB401	E-6	B-16	HP	15	C	18	yes
SB438	E-6	B-16	HP	21	C	18	yes
SB437	E-6	B-16	HP	21	C	18	yes
SB400	E-1	B-7	HP	28	C	25	no
SB410	E-4	B-7	HP	12	C	21	no
SB409	E-5	B-7	HP	15	C	22	no
SB462	E-5	B-7	D	18	C	23	no
SB463	E-5	B-7	D	17	C	24	no
SB465	E-5	B-7	C	13	C	21	no
SB407	E-5	B-7	HP	15	C	22	no
SB421	E-17	B-15	S	1	A	5	no
SB432	E-13	B-8	VC	16	C	27	no
SB464	E-13	B-8	D	17	C	27	no
SB466	E-13	B-8	C	14	C	28	no
SB406	E-5	B-2	HP	23	C	19	no
SB467	E-7	B-17	HP	8	B	16	yes
SB454	E-27	B-23	P	10	B	8	no
SB408	E-11	B-10	HV	1	A	6	no
SB451	E-10	B-10	PF	2	A	7	no
SB413	E-10	B-10	HV	1	A	5	no
SB411	E-10	B-10	HV	3	A	6	no
SB456	E-25	B-22	P	11	B	12	no
SB424	E-22	B-3	VC	30	D	1	no
SB428	E-23	B-3	VC	30	D	1	no
SB427	E-24	B-1	VC	31	D	4	no
SB426	E-8	B-1	VC	4	B	2	no
SB429	E-24	B-1	VC	30	D	4	no
SB431	E-24	B-1	VC	30	D	3	no
SB425	E-9	B-9	VC	32	D	4	no
SB459	E-3	B-18	P	10	B	10	no

inspection of the original gel image. The pattern of strain SB400 was not reproducible even after manual categorization, possibly due to incomplete digestion of a fragment of approximately 9 kb in sample SB400/u, resulting in the low intensity or absence of the 4- and 5-kb fragments (Fig. 1).

Only 5 of 16 pairs of duplicate strains analyzed with *Bam*HI at the two institutes were automatically categorized into the same ribogroup: SB401, SB407, SB410, SB422, and SB438 (Fig. 1). In contrast, manual categorization was reproducible for 16 of 16 duplicates (100%). For the 11 pairs with different outcomes, the nonreproducibility of the RiboPrinter categorization could be attributed to important overall shifts in the patterns, which were more pronounced at high molecular weights, and to the nonreproducibility of bands between 30 and 50 kb (Fig. 1).

In order to investigate whether the poor reproducibility of *Bam*HI automatic categorization could be attributed to poor interinstrument reproducibility or, rather, to an inherent inconsistency of *Bam*HI ribotyping, we analyzed the data obtained with enzyme *Bam*HI after running 40 *E. faecium* strains in duplicate in the UMCU RiboPrinter. This intrainstrument reproducibility analysis was performed with duplicates always being analyzed in distinct batches. Only seven duplicate strains were categorized into the same ribogroup by the RiboPrinter software, whereas manual categorization was reproducible for 38 of 40 duplicates. With regard to interlaboratory comparisons, the nonreproducibility of automated categorization could be clearly attributed to the inherent difficulty of *Bam*HI ribotyping of *E. faecium*, resulting in important overall shifts in the banding patterns in the high-molecular-weight range as well as in a lack of reproducibility of bands larger than 30 kb. The only two instances of nonreproducibility after manual categorization could be attributed to overloading of one of the samples for each duplicate (data not shown).

Discriminatory power of ribotyping. Additional objectives of this study were to evaluate the relative discriminatory power of automated ribotyping by using *Eco*RI and *Bam*HI and to compare the degree of strain differentiation of ribotyping with those of AFLP typing and MLST. Since the reproducibility analysis indicated that for both enzymes, manual ribogroup categorization appeared much more reliable than automated ribogroup categorization, we will hereafter consider only the results of manual categorization. Twenty-eight ribogroups were distinguished by using enzyme *Eco*RI, whereas *Bam*HI ribotyping distinguished 24 ribogroups. When the data obtained with both enzymes were considered in combination, 30 types could be distinguished. In comparison, AFLP typing and MLST distinguished 32 and 28 different types, respectively.

When all four typing methods were considered together, the 49 strains were split into 43 distinct genotypes. In general, *Eco*RI and *Bam*HI ribotyping, AFLP typing, and MLST showed different degrees of discrimination of epidemiologically unrelated strains; e.g., strains discriminated by one method were regarded as identical by another (Fig. 2). Interestingly, ribotyping with *Eco*RI or *Bam*HI achieved the discrimination of some strains that were not distinguished by AFLP typing and/or MLST (Fig. 2). The percentages of strains that were indistinguishable by one typing method but that were found different by another method are given in Table 1 as a quantitative assessment of the discriminatory power and complementarity of these methods. For example, 31% of the strains that were indistinguishable by AFLP typing were distinguished by ribotyping with *Eco*RI, and 38% of the strains that were indistinguishable by ribotyping with *Eco*RI were distinguished by AFLP typing.

The four groups of epidemiologically related strains were characterized as a single ribotype by the two enzymes (Fig. 2). The two epidemiologically linked strains from a hospital in France (SB448 and SB449) were found identical to one epidemiologically nonrelated strain by ribotyping, AFLP typing, and MLST. The three strains from a hospital outbreak in the United Kingdom (SB401, SB437, and SB438) were mutually indistinguishable but were found distinct from all others by ribotyping and MLST but not by AFLP typing. The two strains from the same patient, SB407 and SB409, were found identical to at least one epidemiologically nonrelated strain by ribotyping and AFLP typing but not by MLST. Finally, the U.S. epidemic strains (SB440, SB441, and SB442), which were mutually indistinguishable but were found different from the other strains by both AFLP typing and MLST, were found identical to two other epidemiologically nonrelated strains by ribotyping (Fig. 2).

Identification of genogroups and *esp*-positive strains. The four host-specific genogroups recently identified in *E. faecium* by AFLP analysis (28) were well represented in our study sample (Fig. 2). Genogroup distinction by ribotyping with both enzymes was complete, since in no instance did strains of different genogroups fall into the same ribogroup (Fig. 2). However, differentiation into the four genogroups was not obvious, since the isolates in each genogroup did not cluster on the basis of ribotyping data.

Our study sample included 10 *esp*-positive and 39 *esp*-negative strains, as determined by Southern blotting (27). *esp*-positive strains fell into *Eco*RI ribogroups *Eco*RI-2, *Eco*RI-5, *Eco*RI-6, and *Eco*RI-7 and into *Bam*HI ribogroups *Bam*HI-5, *Bam*HI-6, *Bam*HI-16, and *Bam*HI-17. Of these, only ribogroup *Eco*RI-5 (partly corresponding to *Bam*HI-6) also contained *esp*-negative strains (Fig. 2).

In order to evaluate whether the ribotype data of the 49 strains could be used as a starting ribotype library, the *Bam*HI

FIG. 2. Overview of the patterns obtained after automated ribotyping with *Bam*HI and *Eco*RI restriction enzymes of 49 *E. faecium* strains. Clustering was obtained by using the UPGMA algorithm based on the Dice coefficient calculated from the *Bam*HI patterns. Epidemiologically related strains are indicated by underlining and bold type. SB440, SB441, and SB442 originated from hospital outbreak US-1, and SB401, SB437, and SB438 were from outbreak UK-1 (28). Strains SB448 and SB449 originated from a hospital outbreak in France. Strains SB407 and SB409 were derived from the same patient. The isolation sources of the isolates are indicated by the following abbreviations: P, poultry; PF, poultry farmer; HP, hospitalized patient; D, dog; C, cat; S, swine; VC, veal calf; HV, human volunteer. Strain names, *Eco*RI and *Bam*HI ribogroups, sources, AFLP types, AFLP genogroups, MLST types, and presence or absence of the *esp* gene are indicated in the respective columns. The DNA molecular size scale is given above the fingerprint patterns.

TABLE 1. Comparison of the degrees of strain differentiation by ribotyping, AFLP typing, and MLST

Type	% of strains analyzed by[a]:			
	*Eco*RI	*Bam*HI	AFLP type	Sequence type
*Eco*RI	0	28	31	54
*Bam*HI	8	0	26	38
AFLP	38	46	0	44
Sequence	36	41	23	0

[a] Percentages of strains that were indistinguishable by one typing method but that were found different by another method. For example, 8, 38, and 36% of strains with identical *Eco*RI ribotypes had different *Bam*HI ribotypes, AFLP types, or sequence types, respectively.

patterns of an additional 38 VREF strains (see Materials and Methods) were compared with the present database of the 49 strains and used to identify these new VREF strains. Fifteen different *Bam*HI ribotypes were distinguished among the 38 additional strains. Among these, five ribogroups (comprising 11 strains) were already represented in the database. One of them (*Bam*HI-7) included three hospital strains and were classified as genogroup C (Fig. 2). Two of them (*Bam*HI-10 and *Bam*HI-15) included five strains from healthy individuals and were classified as genogroup A (Fig. 2). Finally, two ribogroups (*Bam*HI-13 and *Bam*HI-24) included three strains from healthy individuals and were classified as genogroup B (Fig. 2). Four additional patterns (comprising five strains) were very similar (one band difference) to patterns already present in the database and were thus presumptively identified at the genogroup level. None of the 38 strains had a ribotype that was identical to ribotypes of the *esp*-positive VREF strains. Likewise, we compared with the study database the *Eco*RI patterns previously obtained for 45 *E. faecium* strains of diverse origins. Strains fell into 39 ribogroups, 7 of which (9 strains) were already in the database. Based on their ribogroups, the assignment of the strains to AFLP genogroups was concordant with the known host specificities of the genogroups. For example, the three strains isolated from chickens fell into ribogroups *Eco*RI-14 (two strains) and *Eco*RI-18 and could thus be classified as genogroup B, and the three strains from hospitalized patients fell into the *esp*-positive strain-containing ribogroups *Eco*RI-2 and *Eco*RI-5 (two strains) and could thus be classified as genogroup C. Seven additional patterns (seven strains) showed a single band difference from the database patterns and were thus presumptively classified at the AFLP genogroup level.

Cluster analysis. We investigated whether distinct ribotype patterns would tend to cluster together according to their genogroups, i.e., whether ribotype patterns reflect the overall resemblance among strains. First, in order to determine the best clustering parameters, we used the panel of duplicate strains to compare the clustering obtained with the whole pattern versus the clustering obtained when we masked the 10% margin at the low-molecular-weight extremity of the pattern (bands smaller than approximately 1.5 kb are excluded from the analysis) and the 15% margin at the high-molecular-weight extremity of the pattern (bands larger than approximately 30 kb are excluded). The Pearson correlation coefficient was used as the similarity index. Better clustering of the duplicate strains was found by use of the second method (data not shown) with both the *Bam*HI and the *Eco*RI data, as expected due to the nonreproducibility of the high-molecular-weight bands and of aberrant bands in the low-molecular-weight range (strain SB441). All subsequent analyses (including band-matching analysis) were performed with the 10 to 85% window.

Clustering results based on band analysis with *Bam*HI ribotyping data are shown in Fig. 2. Similar results were obtained with the Pearson coefficient, but the use of band analysis allowed better visualization of the terminal branches containing a unique ribotype. *Bam*HI clustering showed good agreement with AFLP host-specific genogroups (28). One notable exception was ribotype *Bam*HI-15, which clustered with AFLP genogroup C but was originally identified as belonging to AFLP genogroup A. Also, for the isolates belonging to ribogroups *Bam*HI-1, *Bam*HI-17, *Bam*HI-18, *Bam*HI-23, and *Bam*HI-22, originally assigned to AFLP genogroup B, clustering based on ribotyping and AFLP analysis was not congruent. With one exception (ribogroup *Bam*HI-17), all ribogroups containing *esp*-positive strains clustered in a single branch of the dendrogram. Clustering analysis with *Eco*RI data was also concordant with the AFLP genogroups, although it was less congruent than *Bam*HI clustering analysis.

DISCUSSION

Interlaboratory standardization of bacterial strain fingerprinting is seen as one of the major current limitations of molecular epidemiology (24). Standardized typing technologies are needed to track bacterial clones over large temporal and geographical scales, and the ability to construct large, expandable databases by incorporating data from numerous laboratories would greatly improve knowledge on the epidemiological behavior of important clones. Because automated ribotyping automates most steps of fingerprint data generation and analysis, its major potential advantage is to overcome the difficulty of standardizing experimental parameters; the latter problem limits most widely used molecular typing techniques, including random amplified polymorphic DNA (25), repetitive-element PCR (7), PFGE (26), and AFLP (8). In spite of the commercial development of RiboPrinters in the last few years, no evaluation of the interlaboratory reproducibility of automated ribotyping has been reported in the scientific literature, to our knowledge. In this study, we compared the ribotype fingerprints generated by two distinct RiboPrinters operated in distinct countries by unrelated personnel. Of 35 isolates tested in duplicate with the two instruments, 34 (97%) showed the same ribotype when the patterns were categorized manually. This very high rate of interlaboratory (interinstrument) reproducibility was similar to the rate of intrainstrument reproducibility found in this study (95%). The single instance of nonreproducibility was likely due to incomplete digestion of the genomic DNA. In fact, such incomplete digestion cannot be considered a lack of interlaboratory reproducibility but rather an inherent limitation of the technique itself.

The two instances of intralaboratory nonreproducibility found in this study were most likely due to overloading in one of the duplicate samples, as deduced from the inspection of the raw image. During routine use of the RiboPrinter, such results would be eliminated and the analysis would be repeated with

correct loading. Thus, loading of the samples, which is the only manual step in the generation of automated ribotyping data, can be a source of ambiguity, since it is known that overloading reduces DNA fragment mobility in agarose gels. This effect can be observed mainly in the high-molecular-weight DNA range. Most of the differences between automated categorization and manual categorization can be explained by the fact that visual inspection of the normalized patterns results in intuitively taking into account the overall shift in the patterns due to DNA concentration differences. This intuitive correction is obviously performed by using criteria that are less stringent than those used by the RiboPrinter optimization program. Even though no objective criteria can be proposed for it, intuitive correction of the RiboPrinter categorization is, in our view, necessary and reliable, and intervention of the human eye and judgment after computer analysis of patterns has also been recommended by others (8, 9). The difference in size ranges observed with the enzymes *Eco*RI (2.2 to 9.0 kb) and *Bam*HI (5.5 to 50 kb) can explain the difference in the reproducibility of the two enzymes upon automatic categorization, as the mobility shifts are more pronounced in the high-molecular-weight range.

Another important difference between manual categorization and automated categorization was the window of molecular sizes that was taken into account in the analysis. The window of analysis of the RiboPrinter software ranges from 1 kb to 48 kb (the two extreme molecular weight marker bands); thus, DNA fragments of >30 kb, which are most prone to variations due to concentration differences, are considered in the analysis. In the manual analysis, differences in bands larger than 30 kb are systematically excluded. Analysis of the data after export into BioNumerics software allows correction for this limitation of the RiboPrinter software, as it is possible in BioNumerics software to mask the most nonreproducible zones of the gel during pattern comparisons.

The discriminatory ability of *Eco*RI ribotyping appeared to be similar to that of MLST and only slightly below that of AFLP typing, in spite of the fact that the discriminatory power of AFLP typing compared to that of the other typing methods may have been overestimated because we initially selected strains that had different AFLP types (except for the epidemiologically related groups of strains). In comparison, *Bam*HI ribotyping showed limited discrimination and provided little additional discrimination when considered in combination with *Eco*RI ribotyping. *Eco*RI ribotyping failed to distinguish epidemiologically nonrelated strains, but so did AFLP typing and MLST. It is likely that an epidemiological link existed among these isolates but was not documented. Previously, *Eco*RI ribotyping was compared mainly with PFGE in terms of discriminatory power; the results showed that automated ribotyping has lower discriminatory abilities than PFGE for *E. faecium* (12). Given that the three methods tested here have comparable discrimination and identification capacities, ribotyping could be the method of choice for very rapid analysis of strain relatedness (e.g., in the case of a hospital outbreak), although AFLP typing and PFGE could also be valuable. For larger epidemiological investigations, such as the follow-up of the international spread of clones, standardized technology (ribotyping and possibly AFLP typing) should be used, but MLST would be needed to evaluate the genetic stability of clones and the precise correspondence of ribotypes and AFLP types with clonal lineages. Given that each method discriminated strains that other methods did not discriminate, knowledge of which strains are prevalent in a study area may help in the selection of a first-line method.

The correspondence established among ribogroups, host-specific AFLP genogroups (28), and *esp*-positive strains will render it possible to use ribotyping for genogroup and presumptive *esp* genotype determinations. The identification trials performed in this study are consistent with the host specificity of AFLP genogroups (28). However, the fact that only a small proportion of the strains in the identification trials could be assigned to defined ribogroups based on the initial data set of 49 strains indicates that the number of *Eco*RI reference patterns in the database needs to be increased before a high rate of identification can be achieved. Efforts to pool data collected from several independent RiboPrinters are currently being pursued.

The good correspondence among the AFLP genogroups, sequence types, and ribotype clusters indicates that ribotype patterns, despite the relatively small number of bands, retain much of the information on the genetic relatedness of epidemiologically nonrelated strains with distinct ribotypes. The fact that clustering based on *Eco*RI showed slightly less agreement with AFLP typing and MLST than clustering based on *Bam*HI can be attributed to the faster evolution of *Eco*RI ribotypes, which results in a higher discriminatory power but possibly also concomitantly in the loss of DNA fragments shared among strains within a genogroup.

In conclusion, our results indicate that, even though the RiboPrinter provides automatic categorization of patterns, data must be visually inspected and manually edited before a reliable conclusion can be drawn on the identity or nonidentity of the patterns. Given that *Eco*RI provides better discrimination than *Bam*HI and that interpretation of the *Eco*RI patterns is easier, we recommend *Eco*RI as the enzyme of choice for automated ribotyping of VREF strains. Automated ribotyping necessitates approximately 4 min of hands-on time per strain, does not necessitate highly skilled personnel, and generates results within 8 h. Given these advantages, the high level of discrimination for VREF strains, the high rate of interlaboratory reproducibility, and the potential for epidemiologically important genotype identification, automated ribotyping appears to be a very valuable approach for characterizing VREF strains.

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